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Immunogenicity of a hexavalent combination vaccine in rhesus monkeys

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Abstract

Preclinical immunogenicity studies were conducted in rhesus monkeys to determine whether there is immune interference in the response to one or more components of a hexavalent vaccine (Hexavac™) that contains antigens from *Haemophilus influenzae* (Hib), hepatitis B (HB), diphtheria (D), tetanus (T), acellular pertussis (aP) and inactivated polio virus (IPV). Antibody responses were measured following co-administration of the components at three separate anatomical sites or administration as a hexavalent combination in a single site. After three injections of the hexavalent vaccine, the peak antibody responses to each component of the vaccine were >100-fold above pre-immune titers and persisted at levels >10-fold above pre-immune titers at ≈ 1 year. Immune interference was observed in the peak response to HB, D and pertussis toxin, but was not seen at later time points. The results indicate that the rhesus monkey model may be useful for pre-clinical evaluation of combination vaccines.

Keywords: Combination vaccine; Immune interference; Antigen competition; Non-human primate

1. Introduction

The rationale for the development of combination vaccines has been discussed in recent publications [1–3]. The main benefits are to enhance compliance and vaccine coverage and to reduce overall healthcare costs. An added benefit is to 'make room' in the pediatric vaccination schedule for new vaccines projected for the new millennium [4]. However, experience has shown that the preparation of combination vaccines is far from straightforward. During the development of the DTP combination vaccine, the need for 'balanced' formulations of vaccine components was recognized [5] and careful dose-ranging of the three serotypes of the oral poliovirus vaccine (OPV) was required in order to circumvent interference of the type 2 strain on the immune response to types 1 and 3 [6]. The main impediments to the development of combination vaccines are stability and immunogenicity. Simply mixing existing vaccines can result in incompatibilities among the various antigens, adjuvants, preservatives, stabilizers and excipients, resulting in a loss of stability or reduced potency [7]. A further confounding factor is that of immune interference (also known as antigen competition) which may not always be predicted using animal models. Antigenic competition was first described by Michaelis in 1904 [8], but is still poorly understood.

The objective of the present preclinical immunogenicity studies of Hexavac™ was to determine the antibody response to vaccine component antigens at various times after immunization and to compare the response to the hexavalent vaccine with that induced by administration of Hib, HB and DTaP-IPV at separate anatomic sites. The results indicate that there was a significant difference between experimental and control arms in the peak responses to HB, D and PT. These differences fade with time and there was no significant

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difference in the response to any vaccine component tested at 48–51 weeks post-dose 1. Overall, strong antibody responses were induced to each component of Hexavac™ in rhesus monkeys.

2. Materials and methods

2.1. Experimental animals

The rhesus monkeys (*Macaca mulata*) used in this study were born at the California Regional Primate Center at the University of California at Davis and all immunizations and blood collection procedures were performed at that site. Some monkeys were housed outdoors in social groups, whereas others were maintained indoors, in pairs. Those maintained indoors had a 12:12 h light:dark cycle within a temperature range of ≈17–29°C. Animals were all fed Purina Monkey Chow, 15% protein with fresh produce supplements two to three times per week. Monkeys were identified by tattoos containing unique numbers. The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC).

2.2. Vaccine composition

The hexavalent vaccine consisted of Hib capsular polysaccharide (polyribosyl ribitol phosphate) conjugated to tetanus toxoid (PRP-T), hepatitis B (HB), surface antigen (HBsAg), diphtheria toxoid (D), tetanus toxoid (T), pertussis filamentous hemagglutinin (FHA), pertussis toxoid (PT) and three serotypes of inactivated poliovirus (IPV) formulated with aluminum adjuvants. Each 0.5 ml dose contained 12 μg PRP-T (expressed in polyoside), 5 μg HBsAg, 30 Lf D, 10 Lf T, 25 μg FHA, 25 μg PT, IPV type 1 (40 D-Ag U), type 2 (8 D-Ag U) and type 3 (32 D-Ag U).

2.3. Vaccination/schedule

One group of rhesus monkeys was immunized with half of the human pediatric dose of the hexavalent combination vaccine (Hexavac™) into a single i.m. site, while a second cohort (control group) of monkeys was injected with half of the human dose of PRP-T (ActHIB®), HBsAg (RECOMBIVAX HB®) and DTacP-IPV at three separate i.m. sites at 0, 4 and 8 weeks, according to the protocol shown in Table 1. The monkeys were weighed at each time point and examined for injection site reactions after each dose of vaccine. In addition, blood samples collected at each time point were monitored for changes in white cell, red cell and platelet levels.

2.4. Serological assays

Sera were collected at week 0, 4, 8, 10 and 48–51 and tested individually for antibody titers against PRP, HBsAg, D, T, FHA and PT. Due to a shortage of sera, serology was not performed to detect antibodies against poliovirus. Anti-PRP (component of Hib) antibody titers were measured using a Farr-type radioimmunoassay (RIA), as previously described [9,10]; responses to HB were determined using a modified Ausab® assay (Abbott Laboratories, N. Chicago, IL), as described elsewhere [11]. Antibodies against FHA [12] and antibody titers against T were measured by ELISA [13]. Diphtheria toxoid antibody titers were assayed by using a neutralization test in comparison to a WHO antitoxin standard [14]. PT antibody titers were also determined by a toxin neutralization test on CHO cell culture [15]. The results are expressed as geometric means. The assays used were originally validated for analysis of human samples and adapted for testing monkey serum without further analytical validation.

2.5. Statistical analysis

At each time point, the estimated GMT ratio for the experimental vaccine group relative to the control group (Hexavac™/Hib + HB + DTaP-IPV) and corresponding two-sample 99% confidence interval for the true GMT ratio are calculated for the response to each antigen, assuming unknown but equal variances between the two groups. If a particular interval excludes

Table 1
Protocol for preclinical immunogenicity testing of Hexavac™ in rhesus monkeys

Group	*n*	Age at dose 1 (months)	Vaccine	Injection schedule (weeks)	Bleeding schedule (weeks)
1	8[c]	6–12	Hexavac™[a]	0, 4, 8	0, 4, 8, 10, ≈50
2	8	4.8–10.5	ActHIB®+RECOMBIVAX HB®+DTaP-IPV[b]	0, 4, 8	0, 4, 8, 10, ≈50

[a] Injection volume of 0.25 ml in one intramuscular site.
[b] Injection in three separate intramuscular sites (0.25 ml each).
[c] Serum samples from six of eight monkeys were available at the week ≈50 time point.

the value 1, the corresponding comparison between Hexavac™ and Hib + HB + DTaP-IPV is statistically significant; otherwise, it is not. The reason for using a 99% confidence level instead of the usual 95% level is to control the overall false-positive rate (per antigen), which is defined as the probability that at least one of the confidence intervals will exclude the value 1 by chance alone [16].

3. Results

3.1. Serum antibody response to vaccination

Antibody titers to each component of the vaccine (except IPV) were measured at week 0, 4, 8, 10 and 48–50 using sera from individual animals (Fig. 1). At week 4 (post-dose 1), there were no significant differences between groups in antibody titers to any of the antigens tested. Similarly, at week 8 (4 weeks post-dose 2), there were no significant differences among groups with the exception of the response to HB, which was significantly higher in the control group compared with the monkeys injected with the hexavalent combination. As shown in Table 2, at the 10-week time point (2 weeks post-dose 3), there was a significantly higher response to three of the vaccine components (HB, D and pertussis toxin) in monkeys immunized with separate injections of Hib + HB + DTaP-IPV compared with the response of monkeys immunized with the hexavalent combination vaccine. Importantly, at the final time point (week 48–51), which is 38–41 weeks post-dose 3, there was no significant difference in the response to any component of the vaccines.

3.2. Response rate to vaccination

The percentage of responders to components of the vaccine was determined at each bleed time point. In the absence of established 'seroprotective titers' for rhesus monkeys, the accepted human equivalents were used as shown in the legend to Table 3. For pertussis, there is no proven correlate of protection established for humans, therefore, the percentage of seroconverters was used instead. As shown in Table 3, there was no difference in the response rate to vaccination with Hexavac™ compared with separate site administration of Hib + HB + DTaP-IPV except for the response to HB at the 8-week time point. At that time, only 37% (3/8) monkeys responded to Hexavac™ whereas 100% (8/8) responded to the control. These results are consistent with the analysis of the serological titers that showed a significant difference in anti-HBs titers at this time.

3.3. Adverse event monitoring

Animals were monitored for changes in weight or blood cell counts as well as for injection site reactions. No adverse reactions were noted at the site of injection at any time point and there was no adverse effect of vaccination on the weight or blood cell counts of any animals (data not shown).

4. Discussion/conclusion

The results from the present pre-clinical evaluation of Hexavac™ indicate that there was a vigorous response to each component of the vaccine. Even so, there was evidence for interference in the peak response to HB, D and pertussis toxoid when the responses to Hexavac™ were compared with the control group. Four types of immune interference (antigen competition) have been described: (a) sequential; (b) intramolecular; (c) intravirionic; and (d) intermolecular competition.

- Sequential competition occurs when a second antigen (or vaccine) is given shortly after a first antigen (or vaccine) [17]. This form of interference is especially relevant to vaccine dosing schedules.
- Intramolecular competition results from competition among peptides derived from the same protein for binding to MHC Class I or Class II molecules [18].
- Intravirionic competition results when one protein antigen within a virus interferes with the response to a second protein antigen within the same virus [19]. This form of antigen competition can be circumvented by dissociation of the virus into its component parts prior to immunization.
- Intermolecular competition results when one antigen in a mixture interferes with the immune response to a second antigen in a mixture [17,20]. This form of interference is most relevant to the present investigation; however, the mechanism by which this happens is unknown. One possibility is that one or more components within the combination vaccine become unstable, perhaps due to excipients carried over into the vaccine with a separate component. However, extensive stability studies have been performed on Hexavac™ and the components that had reduced immunogenicity in the combination vaccine (HB, D and PT) were shown to be stable for several years (data not shown). Thus, the decreased response to certain of the vaccine components of Hexavac™ does not appear to be related to a loss of stability of these components, suggesting that the explanation is immune interference due to intermolecular antigen competition.

Although the rhesus monkey model suggests that the response to Hexavac™ is marked by transient interference in response to the HB, D and PT components,

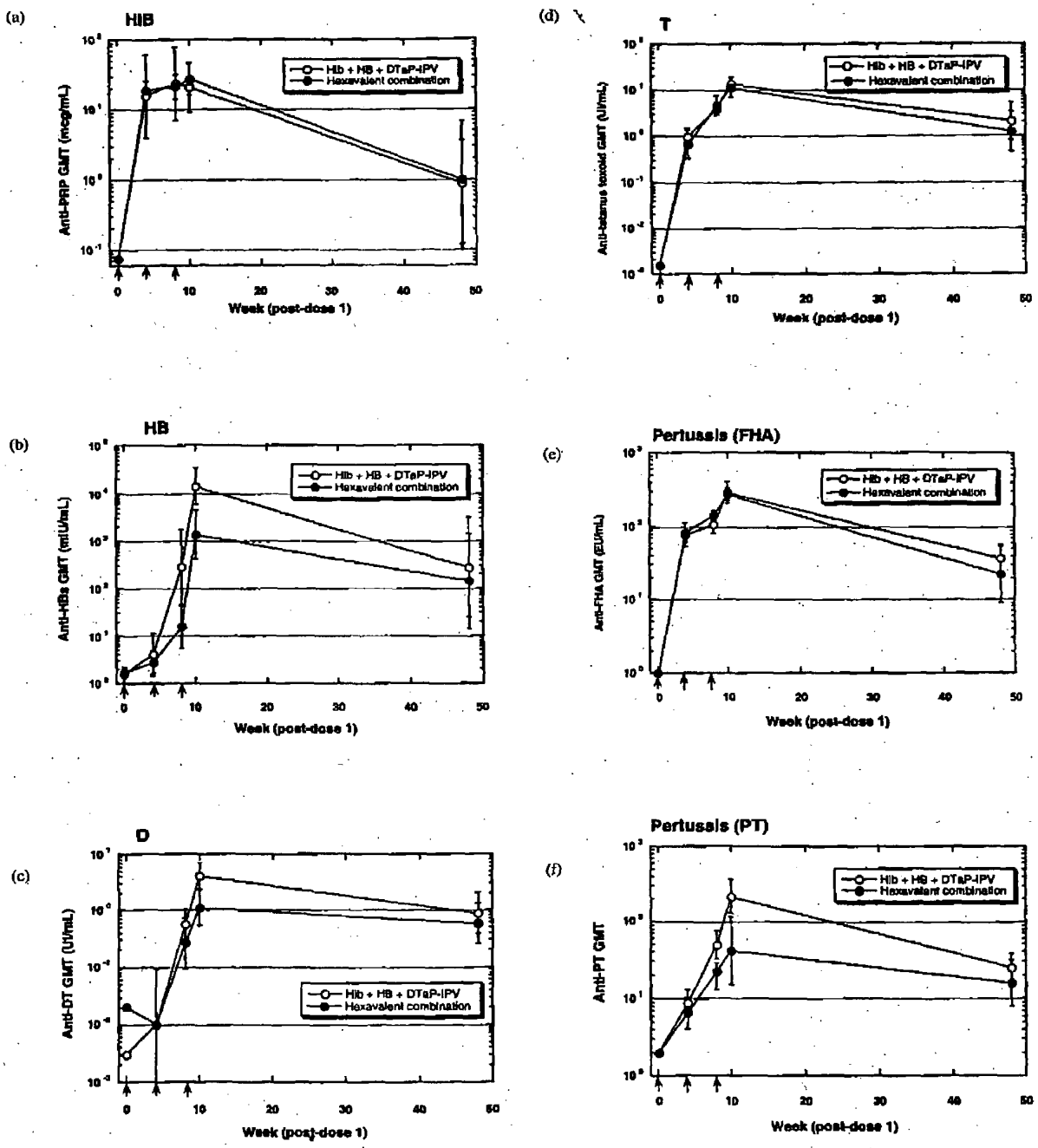

Fig. 1. Antibody response to antigens from Hib, HB, D, T, FHA and PT in rhesus monkeys immunized with Hib + HB + DTaP-IPV at separate sites or with the hexavalent combination vaccine (Hexavac™) at a single i.m. site. Monkeys were immunized at week 0, 4 and 8 and antibody titers were determined on serum collected at week 0, 4, 8 and ≈ 50. Results are expressed as the geometric mean with 95% confidence intervals.

these apparent differences in potency are unlikely to translate into clinically meaningful differences since: (a) the response to each component of the vaccine is > 100-fold higher than the pre-immune titers; (b) the response rate post-dose 3 (percent seroconverters) is equivalent in the two groups; and (c) the difference in titers elicited by Hexavac™ and the control arm became indistinguishable over time. By study week 48–51,

Table 2
Estimated ratios (Hexavac™/Hib+HB+DTaP-IPV) and 99% confidence intervals for the antibody response to Hexavac™ versus control[a]

	Week (post-dose 1)			
Antigen	4	8	10	48–51
HB	0.69 (0.15, 3.21)	**0.06 (0, 0.8)**	**0.1 (0.02, 0.063)**	0.5 (0.01, 37.61)
Hib	1.21 (0.2, 7.16)	0.9 (0.23, 3.56)	0.76 (0.23, 2.56)	2.24 (0.14, 36.74)
D	1.49 (0.09, 25.11)	0.47 (0.1, 2.1)	**0.27 (0.08, 0.86)**	0.66 (0.14, 3.04)
T	0.69 (0.25, 1.95)	1.17 (0.55, 2.49)	0.83 (0.39, 1.79)	0.61 (0.11, 3.4)
FHA	1.08 (0.58, 2.03)	1.31 (0.88, 1.95)	0.94 (0.57, 1.57)	0.6 (0.2, 1.82)
PT	0.77 (0.38, 1.55)	0.46 (0.19, 1.09)	**0.19 (0.05, 0.82)**	0.65 (0.26, 1.64)

[a] Any confidence interval excluding the value '1' implies a statistically significant difference. If the upper confidence limit is <1, then the GMT of Hexavac™ is significantly less than the GMT of Hib+HB+DTaP-IPV. Statistically significant results are in bold type.

Table 3
Response rate to Hexavac™ versus the control group[a]

	Percent responders (Hexavac™, control) at week:				
Antigen	Pre	4	8	10	48–51
HB	0, 0	0, 12	37, 100	100, 100	83, 87
Hib	12, 12	100, 100	100, 100	100, 100	83, 75
D	33, 0	17, 0	100, 100	100, 100	100, 100
T	0, 0	0, 0	100, 100	100, 100	100, 100
FHA	0, 0	0, 0	100, 100	100, 100	100, 100
PT	0, 0	62, 87	100, 100	100, 100	100, 100

[a] Criteria for response: HB (>10 mIU/ml); Hib (>0.15 mcg/ml); D (>0.01 IU/ml); T (>0.01 IU/ml); FHA (>4 UE/ml); PT (reciprocal titer >4).

there was no significant difference in the antibody response to any component of the vaccine between monkeys injected with Hexavac™ versus separate site injection of Hib + HB + DTaP-IPV. The kinetics of the response also deserves comment. With the exception of the response to Hib, the titers to each vaccine component increased following each dose of vaccine. The reason for the immediate and vigorous response to Hib is not known, however, the rapid response suggests that the monkeys may have been primed by prior exposure to *Haemophilus influenzae* or to a cross-reacting organism. Subsequent injections of Hexavac™ did not significantly increase the high titers observed after the first injection, which were > 10 μg/ml. In previous studies [10], 18–22-month-old rhesus monkeys were found to respond earlier and with higher titers to a Hib vaccine (PedvaxHIB®) than 2–3-month-old monkeys. This suggests that the use of younger monkeys may enable better discrimination among Hib-containing vaccines.

The response rate of rhesus monkeys that received three doses of the vaccine was 100% (at week 10). This compares favorably with the response rates seen in a clinical trial of Hexavac™ in which the response rate was 87–100% for each of the antigens [3]. However, the antibody titers achieved in rhesus monkeys were ≈10-fold higher that titers attained in the human post-dose 3. The magnitude of the response of the rhesus monkeys to three doses of Hexavac™ was similar to that of human infants given four injections of Hexavac™ [3]. It should be noted, however, that the small sample size ($n = 8$ monkeys per group) is a limitation in the interpretation of antibody titers and response rates. With these caveats, the rhesus monkey animal model described herein may be useful in the evaluation and development of future combination vaccines.

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References

[1] Ellis RW. Development of combination vaccines. Vaccine 1999;17:1635.
[2] Andre FE. Development and clinical application of new polyvalent combined paediatric vaccines. Vaccine 1999;17:1620.
[3] Pines E, Barrand M, Fabre P, Salomon H, Blondeau C, Wood SC, et al. New acellular pertussis-containing paediatric combined vaccines. Vaccine 1999;17:1650.

[4] Eskola J. Epidemiological views into possible components of paediatric combined vaccines in 2015. Biologicals 1994;22:323.
[5] Barr M, Llewellyn-Jones M. Some factors influencing the response to immunization with single and combined prophylactics. Br J Exptl Pathol 1955;36:147.
[6] Plotkin S, Koprowski H, Stokes J. Clinical trials in infants of orally administrated attenuated poliomyelitis viruses. Pediatrics 1968;23:1041.
[7] Corbel MJ. Control testing of combined vaccines: a consideration of potential problems and approaches. Biologicals 1994;22:353.
[8] Michaelis L. Weitere untersuchungen uber eiweißpruzipitine. Dtsch Med Wschr 1904;30;1240.
[9] Kuo J, Monji N, Schwalbe R, McCoy D. A radioactive antigen binding assay for the measurement for the measurement of antibody to haemophilus influenzae type b capsular polysaccharide. J Immunol Methods 1981;43:35.
[10] Vella P, Staub J, Armstrong J, Dolan K, Rusk C, Szymanski S, et al. Immunogenicity of a new *Haemophilus influenzae* type b conjugate vaccine (Meningococcal protein conjugate) (Pedvax-HIB®). Pediatrics 1990;85:S668.
[11] Wang S, Liu X, Fisher K, Smith JG, Chen F, Tobery TW, et al. Enhanced type I immune response to a hepatitis B DNA vaccine by formulation with calcium- or aluminum-phosphate. Vaccine 2000;18:1227.
[12] Manclark C, Meade B, Burstyn D. Serological response to Bordatella pertussis. In: Rose N, Friedman H, Fahey J, editors. Manual of Clinical Laboratory Immunology, 3rd ed. Washington, DC: American Society for Microbiology, 1986:388.
[13] Bizzini B. Tetanus. In: Germanier R, editor. Bacterial Vaccines. Orlando, FL: Academic Press, 1984:37.
[14] Miyamura K, Nishio S, Ito S, Murata R, Kono R. Micro cell culture method for determination of diphtheria toxin and antitoxin titres using Vero cells: I. Studies on factors affecting the toxin in microplate culture Chinese Hamster Ovary cells. J Biol Stand 1974;2:189.
[15] Gillenius P, Jaatmaa E, Askelof P, Granstrom M, Tiru M. The standardization of an assay for pertussis toxin and antitoxin in microplate culture of Chinese Hamster Ovary cells. J Biol Stand 1985;13:61.
[16] Fisher LD, van Belle G. Biostatistics: A Methodology for the Health Sciences. New York: Wiley, 1983.
[17] Taussig MJ. Antigenic competition. Curr Topic Microbiol Immunol 1973;60:125.
[18] Babbit BP, Matwueda G, Haber E, Unanue ER, Allen PM. Antigenic competition at the level of peptide-Ia binding. Proc Natl Acad Sci USA 1986;83:4509.
[19] Johansson BE, Moran TM, Kilbourne ED. Antigen-presenting B cells and helper T cells cooperatively mediate intravirionic antigenic competition between influenza A virus surface glycoproteins. Proc Natl Acad Sci USA 1987;84:6869.
[20] Pross HF, Eidinger D. Antigen competition: a review of nonspecific antigen-induced suppression. Adv Immunol 1974;18:133.